cRNAs at 1:1:1:1 stoichiometry. After 3 or 4 days of incubation at 22°C (*5*), the oocytes were injected with 200 pmol of EGTA (K^+ salt, pH 7) 1 day before testing the currents. I_{Ba} was measured at 22°C in 40 mM Ba^{2+}, 2 mM K^+, 60 mM Na^+, and 5 mM Hepes (pH 7.4 to 7.5); the anion was either Cl^- or methanesulfonate. A low Ba^{2+} solution containing 2 mM $BaCl_2$, 96 mM NaCl, 2 mM KCl, 5 mM Hepes (pH 7.5) was used in some cases. The two-electrode voltage clamp, data acquisition, and subtraction procedures were as in (*5*). The precision of the rise time measurement was within ±3 ms [N. Dascal and I. Lotan, *Neuron* **6**, 165 (1991)]. The precision of current measurement was usually within ±3 nA. The holding potential was −80 mV. The Ba^{2+}-dependent decrease in I_{Ba} in cells with large currents upon frequent repetitive depolarizations was avoided by using interpulse intervals that allowed a full recovery of I_{Ba} (5 to 40 s, depending on current amplitude).

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Resistance to ddI and Sensitivity to AZT Induced by a Mutation in HIV-1 Reverse Transcriptase

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Serial human immunodeficiency virus type–1 (HIV-1) isolates were obtained from five individuals with acquired immunodeficiency syndrome (AIDS) who changed therapy to 2′,3′-dideoxyinosine (ddI) after at least 12 months of treatment with 3′-azido-3′-deoxythymidine (zidovudine, AZT). The in vitro sensitivity to ddI decreased during the 12 months following ddI initiation, whereas AZT sensitivity increased. Analysis of the reverse transcriptase coding region revealed a mutation associated with reduced sensitivity to ddI. When this mutation was present in the same genome as a mutation known to confer AZT resistance, the isolates showed increased sensitivity to AZT. Analysis of HIV-1 variants confirmed that the ddI resistance mutation alone conferred ddI and 2′,3′-dideoxycytidine resistance, and suppressed the effect of the AZT resistance mutation. The use of combination therapy for HIV-1 disease may prevent drug-resistant isolates from emerging.

EXPERIENCE IN TREATING HIV-1–INFECTED individuals has raised concerns regarding therapy with antiviral agents. Several studies have drawn attention to the emergence of resistant HIV strains in individuals on long-term therapy with AZT alone (*1, 2*). The clinical implications of reduced susceptibility to AZT are not yet fully understood; the appearance of AZT-resistant variants is not associated with a sudden clinical deterioration (*1*). Some individuals who have become intolerant to AZT or appear to be deteriorating clinically have changed therapy to other potentially effective drugs, including ddI (*3*), after discontinuing AZT. To discover if HIV isolates from such individuals develop resistance to ddI and if ddI therapy has any influence on AZT sensitivity we studied sequential isolates from five individuals who had received AZT for at least 12 months and were switched to ddI after they appeared to deteriorate clinically (*4*).

HIV was recovered from peripheral blood mononuclear cells (PBMCs) by cocultivation with donor HIV^- PBMCs (*5*), and drug sensitivity was assessed with a PBMC-based assay (*6*). All isolates recovered at the time therapy was switched had reduced sensitivity to AZT. AZT therapy was discontinued and ddI started (with informed consent). Within 6 to 12 months of discontinuing AZT therapy, the isolates were substantially more sensitive to AZT. During the same time period, a 6- to 26-fold decrease in ddI sensitivity was observed. A representative sensitivity profile for isolates from one individual is illustrated in Fig. 1. After 12 months of ddI therapy, the AZT IC_{50} (50% inhibitory concentration) had dropped from a high of greater than 10 μM to 0.73 μM AZT, whereas the ddI IC_{50}'s for the same isolates increased from 0.3 μM to 9 μM.

Studies have demonstrated that multiple common mutations in the HIV-1 reverse transcriptase (RT) coding region confer resistance to AZT (*7*). Variants with reduced AZT sensitivity were found to contain one or more amino acid changes at codons 67, 70, 215, or 219. To identify mutations in the RT coding region associated with the observed resistance to ddI, we determined the complete nucleotide sequence of the

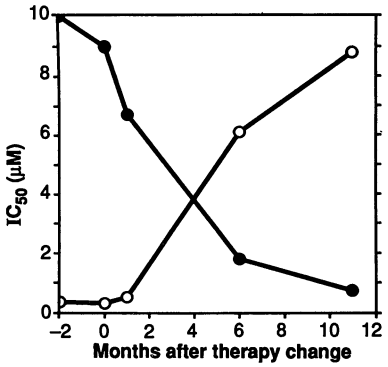

Fig. 1. In vitro sensitivities (IC_{50}'s) to ddI (○) and AZT (●) of sequential HIV isolates from an individual with AIDS. This graph is illustrative of changes seen in five individuals (*4*). At time 0 AZT therapy was discontinued, and ddI therapy (4 mg per kilogram of body mass every 12 hours) was initiated. Blood samples were obtained as shown before, at the time of, and at various times after the change in antiviral therapy. Virus was isolated and analyzed for sensitivity to AZT and ddI as described (*5, 6*).

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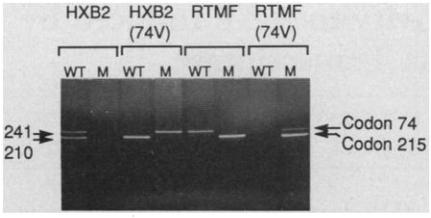

Fig. 2. Selective amplification of DNA fragments from HIV-1 RT used to predict ddI and AZT sensitivity. PCR was used with different combinations of primers, and DNA from MT-2 cells was infected with various HIV-1 variants. For analysis of RT codon 74, we used oligonucleotide primer A (*14*) in each reaction together with primer 74W (wild type, WT) or 74M (mutant, M) to discriminate between wild-type and mutant nucleotides (*11*). For analysis of codon 215, oligonucleotide primer B together with either primer 3W (WT) or 3M (M) was used in PCR as described (7). For each virus, codon 74 and 215 PCR products were combined and analyzed together on a composite tris-borate 3% NuSieve: 1% agarose gel. DNA products from either codon 74 or 215 were distinguished on the basis of size, as the product for codon 74 was 241 bp and that for codon 215 was 210 bp (indicated at left of gel). The HIV-1 variants analyzed, derived from infectious molecular clones, were as follows: HXB2 (wild-type virus), HXB2 (74V) (wild-type HXB2 containing $Leu^{74} \rightarrow Val$), RTMF (HIVRTMF containing $Thr^{215} \rightarrow Tyr$), and RTMF (74V) [HIVRTMF (74V) variant containing $Leu^{74} \rightarrow Val$ and $Thr^{215} \rightarrow Tyr$].

1.7-kb RT coding region from the sequential isolates listed in Table 1. The DNA extracted from cultured PBMCs was amplified by polymerase chain reaction (PCR) (*8*), cloned, and sequenced (*9*). Before the initiation of ddI treatment, the RT from each isolate had the mutation at codon 215 (Thr → Tyr) commonly seen in variants with reduced sensitivity to AZT (7). Additional AZT resistance–associated mutations were also observed in these isolates, namely, $Lys^{70} \rightarrow Arg$ in the isolate from individual 2 and $Met^{41} \rightarrow Leu$ in isolates from individuals 1 and 3 (*7, 10*) (Table 1). Mutations at codons 67 and 219 were not seen in any of these isolates. After initiation of ddI treatment, the mutations at codons 41 and 70 appeared to revert to wild type. However, the isolates obtained from all three individuals retained the mutation at codon 215 as long as 12 months after switching from AZT to ddI. The mutation $Leu^{74} \rightarrow Val$ was seen in the RT of all isolates after 6 to 12 months of ddI therapy and was coincident with decreased sensitivity to ddI (Table 1).

To clarify the role of $Leu^{74} \rightarrow Val$ in resistance to ddI and the mutation's influence on AZT sensitivity, we constructed a series of HIV variants with defined mutations in RT (*11*). The $Leu^{74} \rightarrow Val$ change was introduced into RT by site-directed mutagenesis of the wild-type sequence of proviral infectious clone HXB2-D (*12*) and also of a sequence containing mutations that decrease AZT sensitivity (7). Virus recovered after transfection of DNA clones into T cells was tested for sensitivity to AZT, ddI, and 2′,3′-dideoxycytidine (ddC) by plaque-reduction assay in HeLa-$CD4^+$ cells (*13*). Mutation of $Leu^{74} \rightarrow Val$ caused decreased sensitivity to ddI and ddC both in the wild-type and mutant sequences (Table 2). The magnitudes of these changes were similar to those seen with the clinical isolates. Furthermore, this mutation altered AZT sensitivity only when AZT resistance mutations were also present in RT. For example, variant HIVRTMF (containing mutation $Thr^{215} \rightarrow Tyr$) showed a 20-fold increase in AZT resistance as compared to the wild-type virus (IC_{50}, 0.2 μM). The addition of the $Leu^{74} \rightarrow Val$ mutation caused a decrease in resistance to less than one-tenth the wild-type value (IC_{50}, 0.017 μM). Similar suppression of AZT resistance was observed with variants containing additional combinations of AZT-resistant mutations in RT (Table 2). Although these combinations have not yet been seen in clinical isolates, it is possible they might occur.

We have developed a selective PCR procedure to detect the $Leu^{74} \rightarrow Val$ change (*14*). This enables rapid discrimination of the wild type from mutants at codon 74. Figure 2 shows an example of selective PCR that we used simultaneously to determine the status of codons 74 and 215 in the RT coding region of variants derived from molecular clones. This method could be used to monitor individuals switched from AZT to ddI for the appearance of the $Leu^{74} \rightarrow Val$ change, the only mutation as yet associated with reduced sensitivity to ddI.

This study highlights the value of different techniques used to assess HIV-1 drug sensitivity in vitro. The PBMC assay can be applied to most samples because PBMC cocultivation enables a high rate of HIV isolation. Furthermore, this assay clearly distinguishes sensitivity changes in sequential isolates from the same individual. Direct comparison of absolute IC_{50} values between isolates from different individuals requires careful calculation of input virus because inherent variations in virus replication in vitro can influence assays of this nature (*13*). The HeLa-$CD4^+$ cell plaque assay is useful for quantitating sensitivity changes in isolates from different individuals although it cannot be applied to all samples because only a subset of clinical isolates form plaques in these cells (*1*).

We observed ddI resistance in HIV-1 isolates from individuals with AIDS who switched to ddI after apparent clinical failure with AZT therapy. Mutation at codon 74 in the RT coding region correlated with reduced sensitivity of isolates to ddI. Confirmatory evidence that this mutation was responsible for ddI resistance was obtained by analysis of HIV variants constructed with mutations in RT that mimicked those in clinical isolates. Future studies may reveal additional mutations relevant to ddI resistance. Cross-resistance to ddC was also observed with these variants, raising concerns about the use of ddC after ddI-resistant isolates emerge. The $Leu^{74} \rightarrow Val$ mutation appeared to markedly diminish the effect of a mutation in RT at codon 215 (Thr → Tyr) because AZT resistance decreased despite persistence of the codon 215 mutation. This implies a close interaction between these residues in the active site of RT. Because the mutational basis of decreased viral sensitivity

Table 1. Biologic and genetic properties of HIV clinical isolates and the M13 RT clones derived from those isolates. Sequential HIV isolates from individuals who received AZT and then switched to ddI therapy are shown with the months of ddI therapy at the time the isolates were obtained. IC_{50} values were determined with a PBMC assay (*6*). Amino acid residues relevant to decreased AZT sensitivity (Met^{41}, Lys^{70}, and Thr^{215}) are shown for each isolate. Also shown is the amino acid Leu^{74}, which appears to be associated with decreased ddI sensitivity. Amino acid sequence numbers are relative to the NH_2-terminal proline of the native RT. WT, wild type (HXB2).

Months of ddI therapy	IC_{50} (μM)		Mutations			
	AZT	ddI	Met^{41}	Lys^{70}	Thr^{215}	Leu^{74}
			Individual 1			
−2	>10	0.3	Leu	WT	Tyr	WT
0	9	0.3				
1	7	0.5				
6	2	6	WT	WT	Tyr	Val
11	0.7	9				
			Individual 2			
0	>10	2	WT	Arg	Tyr	WT
5	3	2	WT	WT	Tyr	Ile
12	3	10	WT	WT	Tyr	Val
			Individual 3			
0	6	0.4	Leu	WT	Tyr	WT
5	0.4	0.5	WT	WT	Tyr	WT
11	0.5	3	WT	WT	Tyr	Val

Table 2. Inhibitor sensitivity of HIV-1 variants with altered RTs created by site-directed mutagenesis. Specific nucleotide changes were introduced with synthetic oligonucleotides into the RT coding region cloned into M13. All clones were verified for expression of functional RT in *Escherichia coli*; the maximum variation in RT activity was about twofold relative to the wild-type. Infectious HIV-1 variants were recovered by cotransfection of MT-2 cells with replicative-form M13 DNA containing mutated RT and the RT-deleted provirus clone pHIVΔRTBstEII as described (*11*). The titers of all virus mutants were similar, as determined by plaque assay in HeLa-CD4$^+$ cells (*13*). Sensitivity of these variants to AZT, ddI, and ddC was assessed by plaque-reduction assay in the HeLa-CD4$^+$ cell line HT4-Lac^z (*15*). Mean IC_{50} values derived from at least two independent determinations are shown.

Virus	RT genotype					IC_{50} (μM)		
	Asp^{67}	Lys^{70}	Thr^{215}	Lys^{219}	Leu^{74}	AZT	ddI	ddC
HXB2						0.01	1.8	0.14
HXB2 (74V)					Val	0.01	15	2.2
HIVRTMF			Tyr			0.2	1.8	0.17
HIVRTMF (74V)			Tyr		Val	0.017	12	2.2
HIVRTMC/F	Asn	Arg	Tyr			0.4	1.8	0.1
HIVRTMC/F (74V)	Asn	Arg	Tyr		Val	0.05	48	2.2
HIVRTMC	Asn	Arg	Phe	Gln		1.8	2.5	0.14
HIVRTMC (74V)	Asn	Arg	Phe	Gln	Val	0.06	32	2

is different for AZT and ddI, our findings give further support for the use of multidrug therapy to treat HIV disease. Moreover, combinations of nucleoside analogs directed against RT might be sufficient to prevent resistant variants from appearing.

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8. Frozen PBMCs from cocultures were washed four times with phosphate-buffered saline and lysed (6×10^6 cells per milliliter) in a buffer containing 50 mM KCl, 10 mM tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, porcine gelatin (0.1 mg/ml, Sigma), 0.45% Nonidet P-40 (Sigma), and 0.45% Tween 20. Digestion with proteinase K (60 μg/ml) was performed for 1 hour at 55°C, followed by heat inactivation of the enzyme at 95°C for 10 min. Approximately 1 μg of genomic cellular DNA (25 μl of extract) was used per 100 μl of each PCR mixture containing 50 mM KCl, 10 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 1.0 μM of each oligonucleotide primer, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus). Two primer pairs were required, which allowed four possible combinations, to accommodate sequence variation at the 5′ and 3′ ends of the coding region. The primer sequences were as follows: (5′ end of RT) 5′-TTGCACTTTGAATTCTCCCATTAG-3′ or 5′-GCGCGCATGCGGATCCCCATGGCCATT-AGCCCT-3′ and (3′ end of RT) 5′-CTTA TCTATTCCATCTAGAAATAGT-3′ or 5′-GC-GAAGCTTCTGCAGCTATAGTATTTTCCT-GATTCCAGC-3′. Samples were heated to 94°C for 3 min, and then they underwent 30 cycles of denaturation (30 s at 94°C), primer annealing (2 min at 42° to 55°C, depending on primer pair), and DNA synthesis (3 min at 72°C). Amplification was completed by a final incubation at 72°C for 7 min. PCR products from several reactions were combined and the 1.7-kb band was purified on a gel. We followed recommended precautions [S. Kwok and R. Higuchi, *Nature* **339**, 237 (1989); *ibid.*, p. 490] to prevent PCR contamination, and negative controls were run with all amplification reactions.
9. PCR products were purified electrophoretically on a 1% agarose gel; the 1.7-kb DNA fragments were excised and electroeluted. The fragments were digested with the appropriate restriction enzyme specific for the 5′ and 3′ recognition sites in the amplification primer pair sequences. The fragments were ligated into similarly digested M13mp19 replicative-form DNA. We used the ligation mixture to transform competent *Escherichia coli* (strain JM103) [D. Hanahan, *J. Mol. Biol.* **166**, 557 (1983)]. A library of recombinant M13 clones was selected, and replicative form and single-stranded DNA were prepared from these constructs for nucleotide sequencing. Both strands were sequenced by the dideoxy chain termination method (*12*). We used a set of 11 oligomers (18 nucleotides each) to sequence the entire length of the RT in the replicative form DNA and a set of seven oligomers (20 nucleotides each) to sequence the single-stranded DNA. We also obtained partial sequence information (amino acids 1 to 245) on two additional clones from each isolate to address the issue of sequence diversity and to ensure our samples were representative of the population [J. D. Roberts, K. Bebenek, T. A. Kunkel, *Science* **242**, 1171 (1988)]. Invariant strain-specific mutations were detected from sequential isolates, indicating a high degree of fidelity during PCR primer elongation and nonartifactual cloning of individual PCR products.
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11. HIV variants with defined mutations in RT were generated by cotransfection of a proviral HXB2-D clone (with a deletion in RT) with RT fragments derived from M13 clones. To construct the proviral clone, we first made a specific deletion in M13 clone mpRT1/H, which contains most of the HXB2-D *pol* gene [B. A. Larder, S. D. Kemp, D. J. M. Purifoy, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4803 (1989)]. We used site-directed mutagenesis [M. J. Zoller and M. Smith, *Nucleic Acids Res.* **10**, 6487 (1982)] to delete 1430 nucleotides of the RT coding region between residues 2618 and 4111 of the HXB2-D genome and to simultaneously create a Bst EII restriction enzyme site at the deletion junction by introducing two nucleotide changes (GGTG/ACC). A Bal I fragment containing the RT deletion was excised from the M13 clone, purified from an agarose gel, and ligated with infectious molecular clone HXB2-D (after removal of the full-length Bal I fragment), thus creating the RT-deleted clone pHIVΔRTBstEII. We used M13 clones containing the 1.7-kb HIV-1 RT coding region as the target of site-directed mutagenesis to introduce the Leu^{74} → Val amino acid substitution. These RT fragments were obtained by PCR amplification with the *pol* gene of mpRT1/H as a target, in addition to existing mutants in this clone (7), with an RT-specific PCR primer pair as described (7). Mutations in the RT of M13 clones were verified by nucleotide sequence analysis (*12*), and all mutant clones were checked for expression of functional RT in *E. coli* [B. A. Larder, D. J. M. Purifoy, K. L. Powell, G. Darby, *Nature* **327**, 716 (1987)]. Replicative form DNA was prepared from these clones, and 10 μg of each was digested with Eco RI and Xba I (Bethesda Research Laboratories). This DNA was mixed with 10 μg of Bst EII–digested DNA of clone pHIVΔRTBstEII, and we used the mixture to transfect the T cell line MT-2 by electroporation [A. J. Cann, Y. Koyanagi, I. S. Y. Chen, *Oncogene* **3**, 123 (1988)] using a Gene Pulser (Bio-Rad). The cells were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum and antibiotics. When cytopathic effects were observed because of HIV replication, cultures were expanded by addition of fresh MT-2 cells; after 12 to 14 days virus stocks were prepared from cell-free culture supernatants and stored in aliquots at −70°C.
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14. We performed selective PCR to discriminate wild type (Leu) from mutant (Val) at RT codon 74 and from the mutation at codon 215 as described (7). In brief, 1 μg of DNA extracted from MT-2 cells infected with HIV variants was used as a target for PCR, and two reactions were performed in parallel for each sample with different primer pairs. To identify wild-type codon 74, the primer pair we used was the common oligonucleotide primer A, 5′-TTCCCATTAGTCCTATT-3′, with oligonucleotide primer 74W, 5′-AAGTTCTCTGAAATC-TACTTA-3′. To identify mutant codon 74, we paired the primer A with oligonucleotide primer 74M, 5′-AAGTTCTCTGAAATCTACTTC-3′. PCR conditions were as described for detection of codon 215 mutations (7). PCR products were separated through tris-borate composite gels of 3% NuSieve (FMC BioProducts):1% agarose.
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